〔Full Paper〕

Mutation Screening of the Low-Density Lipoprotein Receptor Gene in Japanese Patients with Familial Hypercholesterolemia from the Kanto Area

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SUMMARY

Forty-one unrelated Japanese patients with heterozygous familial hypercholesterolemia (FH) from the Kanto area (central region) of Japan were screened for mutations in the LDL receptor gene using the polymerase chain reaction (PCR)-restriction enzyme fragment length polymorphism (RFLP) and PCR-heteroduplex analysis methods, followed by sequencing and Southern blotting. Four previously described mutations, 1845+2 T->C (by PCR-RFLP), and D412H, K790X and 327insC (by heteroduplex analysis), were positively identified. In 36 (83.7%) of the patients, mutations were not detected, and the detection rate was lower than previously reported from other regions of Japan. Our results suggest that in the Kanto area, a wide variety of the mutations may be associated with FH, and further refinement of this strategy is required to screen effectively for this disease.

Key words: Low-density lipoprotein receptor, mutation, heteroduplex, PCR-RFLP.

INTRODUCTION

Familial hypercholesterolemia (FH) is a common autosomal dominant disorder that is caused by mutations in the low-density lipoprotein receptor (LDL-R) gene, and is characterized by increased plasma levels of low-density lipoprotein cholesterol (LDL-C), xanthoma, atherosclerosis, and premature coronary heart disease (CHD)[1]. Clinical symptoms develop as cholesterol-rich lipoproteins accumulate in the plasma due to impaired uptake and degradation of LDL by mutant receptors in peripheral cells[1]. CHD can be prevented in FH patients by administering lipid lowering therapy at early stages of the disease. Therefore, early diagnosis of FH by determination of lipid status and genetic screening is clinically important.

Various molecular defects have been identified within the LDL-R gene, including deletions, insertions, missense mutations and nonsense mutations (http://www.ucl.ac.uk/fh/), and to date, a total of more than 800 different mutations have been reported worldwide[2, 3]. The frequency of heterozygous FH is generally estimated to be 0.2% among the general population[3]. In Japanese patients, more than 60 mutations have been reported[4~7]. A limited number of mutations have shown to predominate, and mutations such as 1845+2T->C, K790X and C317S were reported to be common in the Kansai district[4], while five different mutations (K790X, P664L, FH-Tonami-1, IVS15-3C>A and FH Tonami-2) were reported to be frequent in individuals of the Hokuriku district[5]. Based on these reports, it appears that the prevalence of these mutations may vary with regions due to founder effects. However, mutations have not been identified in 30–50% of Japanese FH patients and the familial ligand-defective apoB-100 (FDB), which also causes FH and is common in Europe and North America, is rarely seen in Japan[8]. Therefore, a strategy for detecting known LDL-R mutations, including those common in Japanese individuals, as well as for identifying unknown mutations, is required for effective genetic screening for

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Japanese FH. In the present report, we present the results of screening all exons as well as intron 12 for LDL-R mutations and discuss approaches to establish a rapid and clinically effective genetic screening for FH in the Kanto area.

MATERIALS AND METHODS

Patients

Patients were recruited from the Department of Medicine and Gerontology at Kyorin University School of Medicine and from the Ohmiya Medical Center at Jichi Medical School. A total of 41 unrelated FH patients (16 males, 25 females) and 2 (2 females) of their relatives were included in the analysis. FH was diagnosed based on high plasma levels of LDL-C (>200 mg/dl, 5.2 mmol/L), total cholesterol > 260 mg/dl, 6.7 mmol/L)), Achilles tendon thickness (>10 mm by radiologic examination), and positive family history, according to previously reported criteria[9]. Serum lipid status, including total cholesterol (TC), triglyceride (TG) and high-density lipoprotein cholesterol (HDL-C), was measured by enzymatic methods. LDL-C was calculated according to the Friedewald formula[10].

Isolation of genomic DNA

Genomic DNA was collected from the buffy coat of peripheral blood as previously described[11], and was then suspended in distilled water.

Amplification of genomic DNA

The reported primer pairs for 18 exons[12] and an intron[4] were used. The LDL-R genes of patients were amplified from 1 μg of genomic DNA by polymerase chain reaction (PCR) with 2.5 U DNA polymerase using an automated thermal cycler. The amplification reaction comprised 35 cycles of incubation of the reaction mixture for (1) denaturation at 94°C for 1.5 min, (2) annealing at 60°C to 65°C for 2 min, (3) extension at 72°C for 3 min. PCR products were size fractionated, together with a PCR product from a normal control, by 2% agarose gel electrophoresis. PCR bands for sequencing were isolated from low melting agarose gel and purified using a Prep A Gene kit (Bio-Rad Laboratories, Hercules, CA).

PCR-RFLP

In order to detect mutations in intron 12 (the 1845+2T->C), PCR-restriction enzyme length polymorphism (RFLP) was performed as previously reported[4]. Briefly, a region of the LDL-R gene was amplified from 0.1 μg of DNA by PCR using the exon 12 primer pair, and 10 μl of the amplified product was then digested with BsaHI (Toyobo Co. Ltd., Tokyo, Japan). Restriction fragments were then separated on a 3% agarose gel, stained with ethidium bromide and visualized with ultra violet light.

PCR-heteroduplex analysis

Heteroduplex analysis was performed according to the method of Keen[13]. Briefly, except for exon 12, each of the 17 exon fragments of LDL-R were amplified by PCR. Twenty microliters of PCR product was then mixed with 1 μl of 0.5 M EDTA in a 0.5 ml microcentrifuge tube, and the mixture was exposed to a temperature gradient (94°C to 37°C) using a thermal cycler (PE Applied Biosystems, Foster City, CA) according to the manual provided by the non-denaturing MDE gel manufacturer (AT Biochem, Malvern, PA). Seven microliters of PCR product was mixed with 2 μl of gel-loading buffer and this mixture was loaded onto a 1.6 mm×17 cm×40 cm non-denaturing MDE gel (AT Biochem, Malvern, PA) containing 15% urea in 0.5x TBE, and was separated by electrophoresis at 500 V for 18 h. The homo- and heteroduplexes were visualized by ethidium bromide staining under UV illumination.

Sequencing of genomic DNA

Sequencing of PCR products was performed using an AmpliCycle sequencing kit (PE Applied Biosystems, Foster City, CA) and ^{32}P-labeled primers. We sequenced those cases who showed abnormal PCR-heteroduplex or PCR-RFLP.

Southern blotting

Ten micrograms of genomic DNA was digested by 100 U of BamHI, separated by 0.8% agarose gel electrophoresis, transferred onto a nylon membrane (Biodyne A, Poll) and fixed by UV crosslinking. Membranes were then subjected to hybridization and autoradiographic detection with ^{32}P-labeled LDL-R cDNA probes for exons 2 to 18 (2.4 kb of RT-PCR product of LDL-R mRNA from a human cell line).

RESULTS

Detection of the 1845+2 T->C mutation by PCR-RFLP

Figure 1 shows the PCR-RFLP results of 5 individuals. PCR products of intron 12 from a normal control after BsaHI digestion gave a single 190-bp band (lane NC). On the other hand, PCR products from a patient (Lane H.H.) yielded two bands (166 bp and 190 bp), indicating that the mutant allele was heterozygous in this patient. Bands in the rest of the patients (Lane 3–5) were the same as those seen in the normal control (Figure 1). However, we identified an other patient (K.K., unrelated to H.H.) who showed same abnormal pattern.

Heteroduplex analysis and confirmation by DNA sequencing

Heteroduplex analysis of 18 exons of the LDL-R gene in 41 unrelated Japanese hypercholesterolemic patients gave aberrant heteroduplex bands in 4 patients. The hetero-

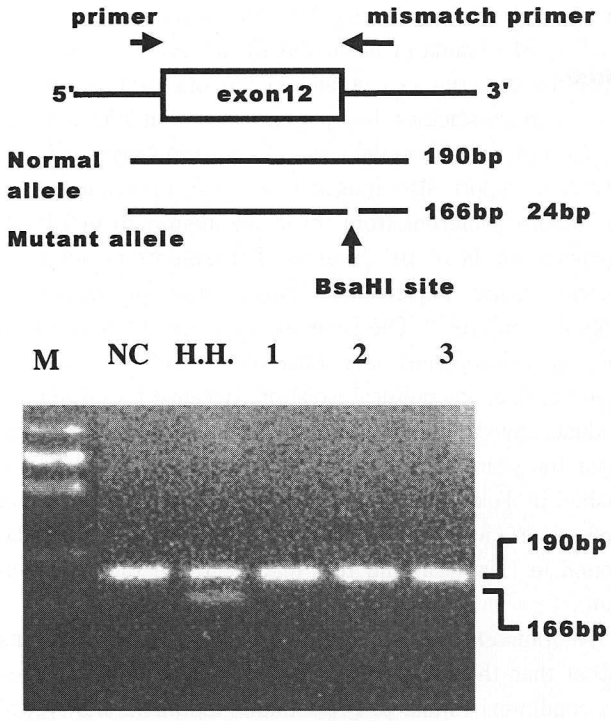

Fig. 1. Shematic presentation of restriction map and detection of 1845+2T->C mutation by PCR-RFLP.

The mutation 1845+2T->C results in a BsaHI cutting site in exon 12 of the LDL-R gene (upper). The PCR product of intron 12 after BsaHI digestion from a normal control showed a single 190-bp band (lane NC). The PCR products from a patient yielded two bands, 166 bp and 190 bp (shown in Lane H. H.) . Lanes 1, 2 and 3 are the PCR products from three FH patients, but have a normal pattern (lower).

duplex band of exon 4 in one patient (H.W.) showed abnormal patterns (Figure 2, A, upper)[7]. DNA sequencing of the mutant heteroduplex bands indicated that exon 4 from patient H.W. had undergone a single base insertion (C) at codon 109 that shifted the reading frame and introduced a premature termination codon (TGA) following amino acid residue 49 (codon 158) in the ligand binding domain of the LDL-R gene (Figure 2, A, lower), whereas his wife and daughter were normal (data not shown). The heteroduplex band of exon 9 of one patient (G.S.) was abnormal (Figure 2, B, upper). Sequencing of exon 9 from the patient showed a single base substitution (G to C) at position 412 resulting in a missense mutation (Asp to His) (Figure 2, B, lower). The heteroduplex band of exon 17 of two patients (S.H. and J.M.) was also abnormal (Figure 2, C, upper). Sequencing of exon 17 from patients S.H. and J.M. showed a single base substitution (A to T) resulting in a stop codon (TGA) in place of lysine (K790X in Figure 2, C, lower). These results also indicated that the patients were heterozygous for the mutant allele. There were no differences regarding heteroduplex pattern of normal control DNA and that from the other patients.

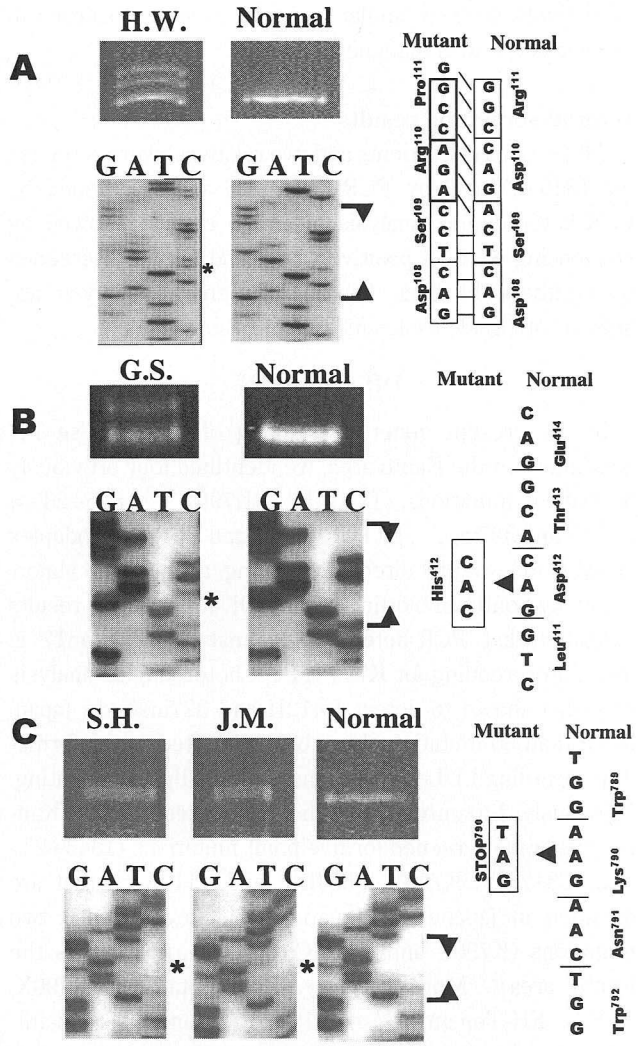

Fig. 2. Detection of mutations by PCR-heteroduplex analysis and nucleotide sequencing.

A: The heteroduplex band of exon 4 from one patient (H.W.) showed abnormal patterns with two additional bands (upper). The DNA sequence of exon 4 from patient H.W. revealed a single base insertion (C; indicated by*) at codon 109 that shifted the reading frame and introduced a premature termination codon (TGA) following amino acid residue 49 (codon 158) (327insC), whereas those from normal control showed normal sequences (lower)[7].

B: The heteroduplex band of exon 9 from a patient (G.S.) was abnormal (upper). Sequencing revealed a single base substitution (G to C) resulting in a missense mutation (Asp→His) at the position 412 (lower) (D412H).

C: The heteroduplex bands of exon 17 from two patients (S. H. and J.M.) were abnormal (upper). The sequences showed a single base substitution (A to T) resulting in a stop codon (TAG) in place of lysine (K790X) (lower)

Southern blot analysis using LDL-R cDNA

The genomic DNA from patient M.H. after BamHI digestion gave two bands of approximately 20 and 10 kb. On the other hand, the genomic DNA from a normal control yielded a single band of approximately 10 kb, suggested a heterozygous BamHI site deletion in the genome of the patient M.H.. The genomic DNA from the remaining

15 patients showed similar patterns as were observed in the normal control (data not shown).

Overall screening results

All 43 cases (41 patients and two relatives) were screened for 1845+2T->C by PCR-RFLP, 36 were screened by PCR-heteroduplex analysis of all 18 exons, followed by sequencing of the 4 positive cases, and 16 were screened by southern blotting. Genetic screening positives are shown with lipid profiles in Table 1 (Table 1).

DISCUSSION

In the present genetic screening of 43 Japanese FH patients from the Kanto area, we identified four previously described mutations, D412H[11], K790X[4], 1845+2T->C[14,15] and 327insC[7], by PCR-RFLP and PCR-heteroduplex analysis followed by direct sequencing, and a single abnormality by Southern blotting in the LDL-R gene. Our results indicated that PCR-heteroduplex analysis of exon17 is useful in screening for K790X. PCR-heteroduplex analysis was also shown to detect D412H and 327insC. In Japan, more than 60 mutations have been reported, and information regarding LDL-R mutations is gradually accumulating. Previously, 120 unrelated FH heterozygotes from the Kansai area were screened for five point mutations (1845+2T->C, C317S[15], K790X, P664L[12] and E119K[12]) that are common in Japanese FH patients[4]. They included two mutations (K790X and E119K) whose birth place is the Kanto area[4]. More recently, five mutations (K790X, P664L, FH-Tonami-1[16], IVS15-3C>A and FH-Tonami-2[16]), of which two overlapped with the above report, were found to be common in 200 FH patients from the Hokuriku area[5]. The researchers used PCR denaturing gradient gel electrophoresis (DGGE), DNA sequencing and Southern blotting. Using similar methods, another group reported several common mutations (including 6 unidentified mutations and 3 previously reported mutations; C317S, K790X and 1845+2T->C) in 120 FH patients in the Kanto area[6]. Their detection rate of 14/120 (11.6%) in the Kanto area is similar to that of the present study, which had a detective rate of 5/41 (12.2%). These were lower than reported in previous studies by Yu et al. (41.5%) in the Hokuriku area and by Maruyama et al. in the Kansai area (31.7%), and thus the detection rate obtained using our strategy may be less than satisfactory. In Japan, numerous additional mutations, including large deletions, have been reported[17~23]. Another report also indicated a lower prevalence of 7 mutations (different from those we identified) in LDL-R genes from 44 of 101 Japanese FH patients of unknown origins using single-strand conformation polymorphism (SSCP) analysis[24]. The Japanese are generally believed to be a genetically uniracial population due to their prolonged geographical and political isolation. Because Japanese individuals have been moving to the Kanto area for at least the past 100 years, since the national capital was firmly established in Tokyo (center of Kanto area), a wide variety of mutations, including those originating locally, may be found in this area, and this may decrease the detection rate.

Another explanation is the fact that genetic mutations other than those in LDL-R clinically manifest themselves as conditions similar to FH. Genetic mutations not typical of those commonly observed in FH and that result in FH-like symptoms may remain undetected in our group. Sitosterolemia is such a condition[25]. This is an FH-like disease that is caused by mutations in two adjacent ATP-binding cassette (ABC) transporters, ABCG5 and ABCG8, and is characterized by elevated plasma plant sterol and cholesterol[25]. The frequency of sitosterolemia among Japanese FH patients remains unclear. Other candidate diseases include a condition linked to chromosome 1p34-32 that is associated with autosomal dominant hypercholesterolemia[26]. These conditions could not be excluded in our study. However, the familial ligand-defective apoB-100 (FDB), which also causes FH and is common in Europe and Noth America is rarely found in Japan[27].

The low detection rate in the current study may be due to the fact that numerous LDL-R and related mutations, including common mutations, remain undetected by our strategy. With regard to methodology, the characteristics of the method selected may affect the results of screening[29~31]. Therefore, targeting of all 6 mutations

Table 1. Clinical data for Japanese FH patients whose mutations were identified in this study.

Patient	Sex	Mutation site	TC	TG	HDL-C	LDL-C	IHD	Xanthoma	Ref
H.H.	F	1845+2T->G	323	95	36	268	−	+	14,15
K.K.	M	1845+2T->G	340	121	40	276	−	+	14,15
H.W.	M	327insC	428	178	44	348	−	+	7
G.S.	M	D412H	323	156	22	270	+	−	11
S.H.	F	K790X	428	99	33	375	+	−	4
J.M.	M	K790X	386	59	53	321	−	−	4
M.H.	F	UN (deletion?)	318	143	44	245	−	−	

TC, Total cholesterol; TG, Triglycerides; HDL-C, High-density lipoprotein cholesterol; LDL-C, Low-density lipoprotein cholesterol; IHD, Ischemic heart disease; UN, Unknown.

common in Japanese patients (C317S, P664L, E119K, FH-Tonami-1, IVS 15-3C>A and FH-Tonami-2), should have been included in the strategy for the Kanto area. In one patient, we detected a heterozygous deletion of the BamHI cutting site, but we were unable to conclusively determine the cause. The database search of genomic LDL-R (19p13.3) from NCBI's web site (http://www.ncbi.nlm.nih.gov/genome/guide/human/), by using DNASIS-MAC ver.3 (Hitachi software engineering) shows that five DNA fragments (14.9 kb, 16 kb, 2.2 kb, 2 kb, 0.1 kb) that can bind to the probe for exons 2 to 18, are produced by BamHI digestion of human cDNA, Two of the predicted bands (14.9 kb, 16 kb) seemed to correspond to a single 10 kb band of the normal control yielded in our study. Because the genomic DNA from patient M.H. after BamHI digestion gave an extra 20 kb band, it was suggested that any one of BamHI sites between genome No. 32718 and 76075 was deleted in the genome of the patient. Technological innovation may ensure that future screening is reliable and convenient. DNA chips or solution-phase microarrays[31] are potential candidates, if cost per sample can become acceptable. In conclusion, we herein presented the results of genetic screening in Japanese FH patients and discussed approaches to establish an effective screening strategy that is applicable to the Kanto area.

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